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Interaction of Enteric Bacterial Pathogens with Murine Embryonic Stem Cells▽†

Jun Yu,*‡ Raffaella Rossi,‡ Christine Hale, David Goulding, and Gordon Dougan

The Wellcome Trust Sanger Institute, The Wellcome Trust Genome Campus, Hinxton, Cambridge CB10 1SA, United Kingdom

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Embryonic stem (ES) cells are susceptible to genetic manipulation and retain the potential to differentiate into diverse cell types, which are factors that make them potentially attractive cells for studying host-pathogen interactions. Murine ES cells were found to be susceptible to invasion by *Salmonella enterica* serovar Typhimurium and *Shigella flexneri* and to the formation of attaching and effacing lesions by enteropathogenic *Escherichia coli*. *S. enterica* serovar Typhimurium and *S. flexneri* cell entry was dependent on the *Salmonella* pathogenicity island 1 and *Shigella mxi*/*spa* type III secretion systems, respectively. Microscopy studies indicated that both *S. enterica* serovar Typhimurium and *S. flexneri* were located in intracellular niches in ES cells that were similar to the niches occupied in differentiated cells. ES cells were eventually killed following bacterial invasion, but no evidence of activation of classical caspase-associated apoptotic or innate immune pathways was found. To demonstrate the potential of mutant ES cells, we employed an ES cell line defective in cholesterol synthesis and found that the mutant cells were less susceptible to infection by *Salmonella* and *Shigella* than the parental ES cells. Thus, we highlighted the practical use of genetically modified ES cells for studying microbe-host interactions.

Cellular microbiology has emerged as an important field that contributes significantly to our understanding of the interactions between the host and the pathogen during an infection. Conventionally, terminally differentiated cells, such as epithelial cells, macrophages, and lymphocytes, have been exposed to microbes and their products in studies to decipher the host cellular response to potentially infectious agents (9). Controlled exposure of cultured host cells to microbes has provided insights into underlying mechanisms of pathogenesis and immunity (8). We are now entering a postgenome era in which the complete genome sequences of many pathogens, as well as hosts, including mice and humans, are available, and major advances in our understanding of infectious diseases will inevitably be powered by exploiting this information (2, 6).

Mice have a long history of exploitation as fruitful model mammals for studying a variety of infectious diseases. The sequencing of the mouse genome has revealed that the majority of the genes of mice and humans are shared, further justifying the use of murine systems as genetic models for human infections (38). Furthermore, the wealth of expertise in mouse genetics and mutagenesis facilitates the manipulation of host genes (33) in a way which would not be ethically feasible with human cells. Several new resources are becoming available that should allow genome-wide screens to be performed in murine cells. Thousands of mutant embryonic stem (ES) cell lines harboring randomly inserted gene traps (36; www.genetrap.org) or in which murine genes have been directly targeted are being generated with the eventual aim of inactivating all known mouse genes over the next decade (7; www.eucomm.org; www.knockoutmouse.org; www.norcomm.phenogenomics.ca; www.sanger.ac.uk). Mutant ES cell lines obtained from these resources can be propagated as immortal stem cell lines, differentiated into different cell types (14, 15), or even used to produce mutant mice for studying disease pathology in vivo. Random mutagenic screens with murine cells are becoming feasible based on the observation that there is an elevated loss of heterozygosity during mitosis in Bloom's (Blm) syndrome protein-deficient ES cells (28), and prototype screens have led to the identification of novel genes involved in DNA mismatch repair (20).

In order to investigate whether ES cells can be used directly to explore host-pathogen interactions, we assessed the responses of readily available murine ES cells following exposure to different bacterial pathogens, including *Salmonella enterica* serovar Typhimurium, *Shigella flexneri*, and enteropathogenic *Escherichia coli* (EPEC). The significant findings of this study are presented here.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *S. enterica* serovar Typhimurium SL1344 is a fully virulent *Salmonella* strain, whereas SL1344(pSsaG) is a derivative that directs the expression of green fluorescent protein (GFP) from the *ssaG* promoter (31). *S. flexneri* M90T is a virulent *Shigella* strain, while the *mxiD* and Sh53 mutants (J. Yu, unpublished) are defective in the type III secretion system (TTSS) translocon and periplasmic peptidylprolyl isomerase, respectively. *E. coli* 31-6-1 is an enteropathogenic and locus of enterocyte effacement-positive strain with a bundle-forming pilus mutation (11). Most bacteria were routinely grown on Luria-Bertani agar (L agar) plates at 37°C; the exceptions were the *S. flexneri* strains, which were grown on L agar plates containing 0.01% Congo red. Antibiotics were added at the following concentrations when they were required: ampicillin, 100 μg/ml; and kanamycin, 50 μg/ml. Specific culture conditions for experiments are described below.

ES cell lines and cell culture conditions. NN5 is a $Blm^{-/-}$ ES cell line derived from AB2.2 (D. Adams, unpublished). TBV2 is a wild-type ES cell line from a

* Corresponding author. Present address: Microbiology, Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde Royal College, 204 George Street, Glasgow G1 1XW, Scotland. Phone: 44-141-548-2137. Fax: 44-141-553-4124. E-mail: jy1@sanger.ac.uk.

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‡ J.Y. and R.R. contributed equally to this work.

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129S2 mouse embryo, and M064/F03 was derived from TBV2 bearing a gene trap in the Sqle gene required for cholesterol synthesis. All cell lines were routinely cultured in gelatin-treated flasks or plates under a humidified atmosphere containing 5% CO_2 at 37°C in complete Glasgow's minimal essential medium (GMEM) (Sigma) supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 1× nonessential amino acids, 10% (vol/vol) fetal bovine serum (FBS), a 1:1,000 dilution of a β-mercaptoethanol stock solution, and 500 to 1,000 U per ml of leukemia-inhibiting factor (LIF). Most supplements were purchased from Invitrogen; FBS was purchased from HyClone, and LIF was purchased from Chemicon.

Infection of ES cells with *S. enterica* serovar Typhimurium and *S. flexneri*. ES cells were seeded into either 24-well plates (0.5×10^5 cells per well) or six-well plates (2.5×10^5 cells per well) and cultured for 2 days. Salmonellae were initially cultured at 37°C with shaking (250 rpm) in 5 ml L broth for 4.5 h. An aliquot was then diluted 1:50 in L broth and grown at 37°C overnight as a static culture to optimize *Salmonella* pathogenicity island 1 (SPI1) gene expression. *S. flexneri* cultures were grown in L broth overnight at 37°C and then diluted 1:50 and grown at 37°C with shaking (250 rpm) for 2 to 3 h prior to use. For infection, the optical density at 600 nm of bacterial cultures was adjusted to between 0.6 and 0.7, and then suspensions in fresh GMEM containing 10% (vol/vol) heat-inactivated FBS were prepared to obtain a multiplicity of infection (MOI) of either 10 or 100 as indicated below. The MOI was confirmed by plating 10-μl spots of 10-fold serial dilutions of the bacterial stock in triplicate onto L agar plates and incubating the plates overnight at 37°C. To facilitate *Shigella* invasion, the cell culture plates were spun for 10 min at 2,000 rpm at room temperature. After 30 min of incubation (to allow *Salmonella* or *Shigella* invasion), cells were washed with phosphate-buffered saline (PBS) before complete GMEM supplemented with gentamicin (50 μg/ml) was added. Cells were incubated for the appropriate length of time and then washed and lysed with 0.1% Triton X-100. Dilutions of the cell lysates were plated onto L agar plates to determine the number of intracellular bacterial CFU. Alternatively, cells were trypsinized and fixed with 1% paraformaldehyde for flow cytometry.

Infection of ES cells with EPEC. ES cells were cultured on coverslips in six-well plates as described above for 2 days. Bacterial subcultures were prepared by 1:100 dilution of an overnight culture in GMEM and grown for 3 to 4 h at 37°C with shaking (250 rpm). One milliliter of a bacterial culture was utilized to replace the spent medium in a well and incubated for 3 to 4 h under an atmosphere containing 5% CO_2 at 37°C. The cells were washed with PBS to remove the unattached bacteria and fixed with 3.7% formaldehyde in PBS for 20 to 30 min at room temperature before they were processed for fluorescent microscopy.

Fluorescent microscopy. For observation of attaching and effacing lesions induced by extracellular bacteria, cells infected with EPEC (which was transformed with GFP plasmid pJKD18) were fixed with 3.7% formaldehyde for 20 to 30 min at room temperature, permeabilized with 0.1% Triton X-100 in PBS for 5 min, and stained to reveal actin with Texas Red phalloidin (1 U in 300 μl of PBS) for 20 min at room temperature. Coverslips were washed twice with PBS and mounted onto glass slides with ProLong Gold antifade reagent (Invitrogen) containing 4′,6′-diamidino-2-phenylindole (DAPI) for nuclear staining. The preparations were observed with an LSM510 META confocal microscope (Zeiss).

For observation of intracellular *Salmonella* and *Shigella*, cells were washed twice with 50 mM H_4NCl in PBS after fixation and permeabilized with 0.1% Triton X-100 in PBS for 5 min. The cells were blocked with 5% bovine serum albumin in PBS for 30 min at room temperature and stained with appropriate primary and secondary antibodies as indicated below. To observe M90T(pJKD18)-directed actin tails, cells were treated as described above and stained with Texas Red phalloidin as described above for EPEC. To observe apoptosis, ES and J774 cells were infected with *S. enterica* serovar Typhimurium SL1344(pSsaG) (MOI, 100) for 2 h. The cells were washed with PBS and stained with sulforhodaminyl-L-valylalanylaspartyl fluoromethyl ketone (SR-VAD-FMK) (Immunochemistry Technologies) at 37°C under an atmosphere containing 5% CO_2 for 1 h. The cells were then washed and fixed with 3.7% formaldehyde for 20 min at room temperature. Glass coverslips were mounted with ProLong Gold antifade reagent (Invitrogen) for microscopy.

Cholesterol was visualized by exposing fixed cells to the filipin complex, diluted to obtain a concentration of 0.05 mg/ml in PBS containing 5% FCS, for 2 h before they were washed three times with PBS. Further internal staining was performed after permeabilization of the cells.

Electron microscopy. Scanning electron microscopy (SEM) was performed as follows. After infection with *S. enterica* serovar Typhimurium SL1344 or *S. flexneri* M90T for 30 min (MOI, 100), cells were fixed directly on glass coverslips with 2.5% glutaraldehyde and 4% paraformaldehyde in 0.01 M PBS at 4°C for 1 h, rinsed thoroughly in 0.1 M sodium cacodylate buffer three times, and fixed again in 1% buffered osmium tetroxide for 3 h at room temperature. To improve conductivity, using the protocol devised by Malick and Wilson (29), the samples were then impregnated with 1% aqueous thiocarbohydrazide and osmium tetroxide layers, with the steps separated by sodium cacodylate washes. The coverslip preparations were dehydrated three times using an ethanol series (30, 50, 70, 90, and 100% ethanol, 20 min each) before they were critical point dried in a Bal-Tec CPD030 and mounted on aluminum stubs with conducting araldite. Before a specimen had completely set, the coverslip was broken by applying pressure with a sharp point to the center, which caused radial fragmentation of the glass, in order to obtain better conductivity between the stub and the cells. Finally, the coverslip was sputter coated with a 2-nm gold layer in a Bal-Tec SCD050 and examined with an Hitachi S-4800 SEM. For transmission electron microscopy (TEM), cells were infected as described above and fixed on ice in their culture wells with a mixture of 2.5% glutaraldehyde and 4% paraformaldehyde in PBS for 1 h. The cells were rinsed three times with 0.1 M sodium cacodylate buffer (pH 7.42), carefully removed from the plate with a Teflon scraper, and centrifuged at 10,000 rpm for 5 min. The pellet was postfixed in buffered 1% osmium tetroxide at room temperature for 1 h, followed by 1% buffered tannic acid for 30 min and then a 1% aqueous sodium sulfate rinse for 10 min. Finally, the sample was dehydrated using an ethanol-propylene oxide series (with 2% uranyl acetate added at the 30% step) and embedded in Eponaraldite for 24 h at 60°C. Ultrathin sections (60 nm) were cut with a Leica EMUC6 ultramicrotome, contrasted with uranyl acetate and lead citrate, and viewed with an FEI 120-kV Spirit Biotwin TEM. Images were obtained with a Tietz F415 digital TemCam.

Flow cytometry. After trypsinization and fixation with 1% paraformaldehyde in PBS, cells were analyzed with a BD FACSAria (BD Biosciences) for intracellular GFP (fluorescein isothiocyanate [FITC]) signals in the case of *S. enterica* serovar Typhimurium SL1344(pSsaG) and SL1344(pJKD18) and *S. flexneri* M90T(pJKD18) or their mutant forms. To detect Oct3/4 and CD11b, cells were stained with 1:50 dilutions (in PBS containing 5% FBS and 0.01% sodium azide) of appropriately conjugated (with phycoerythrin [PE] and allophycocyanin [APC], respectively) antibodies for 15 min in the dark. For Oct3/4 staining the cells had to be fixed prior to permeabilization with 0.1% Triton X-100 in PBS for 5 min.

Real-time RT-PCR. Total mRNA (1 μg) from either ES cells or J774 cells was used to synthesize cDNA (QuantiTech reverse transcriptase kit; Qiagen), and one-tenth of the resulting total cDNA was utilized for real-time reverse transcription-PCR (RT-PCR) with a SensiMixPlus SYBR (Quantace) kit using a Stratagene Mx3000 RT machine. The samples were run in triplicate using the manufacturer's recommended protocol, and the results were recorded as mean cycle threshold (C_T) values. The mean C_T is the minimum number of cycles at which PCR products were detected. Using the delta-delta method developed by Perkin Elmer (Applied Biosystems), the mean C_T was used to calculate ΔC_T values with the following formula: ΔC_T = mean $C_T^{\text{target gene}}$ − mean $C_T^{\text{control gene}}$. The normalized mean ΔC_T was obtained by subtracting the mean C_T of the target gene from the mean C_T of the internal control β-actin gene. $\Delta\Delta C_T$ values were calculated by subtracting the ΔC_T value of the target gene in the control (J774) cell line from the ΔC_T value of the same gene in the target (ES) cell line. The $\Delta\Delta C_T$ values were expressed as follows: ln 2^(−$\Delta\Delta C_T$), where 2 reflects the RT-PCR efficiency.

Primers. The following primers were used: for caspase-1, 5′TTTCAGTAGC TCTGCGGTGT and 3′TTTCTTCCTGATTCAGCACTCTC; for caspase-3, 5′TACTGCCGGAGTCTGACTGG and 3′TCCTCATCAGTCCCACTGTCT; for caspase-9, 5′ACGCTCTGCTGAGTCGAGA and 3′GGTCTCAAGGTCTG TGACCA; for Sqle, 5′CCTGTTGGGTTGCTTTCAAT and 3′CACGTGGAC TCCCTTTCAAT; and for β-actin, 5′AGGCCAACCGTGAAAAGATG and 3′CACAGCCTGGATGGCTACGT.

Affymetrix microarray analysis. Total RNA was obtained from ES cells that were not infected and were infected with *S. enterica* serovar Typhimurium SL1344(pSsaG) as described by McKelvie et al. (31), using an RNeasy mini kit (Qiagen). The criteria for total RNA extraction were such that at least 30% of the cells were infected at 2 or 4 h postinfection, as judged by a parallel flow cytometric analysis. The total RNA concentration was determined with a NanoDrop 1000 (Thermo Fisher Scientific), and the quality was verified with a Bioanalyzer (Agilent). cDNA was synthesized using a one-cycle cDNA synthesis protocol and was labeled with biotin using a GeneChip IVT labeling kit according to the Affymetrix protocol for eukaryotic cells (GeneChip expression analysis technical manual; Affymetrix). Three technical replicates were used for uninfected and infected cells at 2 and 4 h postinfection, and three independent experiments were carried out for biological reproducibility. The quality of the labeled cDNA was verified by Bioanalyzer analysis prior to hybridization. An

Affymetrix GeneChip mouse 430 2.0 array (with 45,000 probes sets representing more than 35,000 mouse genes) was hybridized with the biotin-labeled cDNA overnight at 45°C. The chips were washed and stained with an Affymetrix Fluidics Station 450, and a GeneChip Scanner 3000 was used to capture the signals. The microarray analysis was performed utilizing GeneSpring version 7.3.1 software. The raw data were subjected to quality control analysis as suggested by Affymetrix and then normalized using the GeneChip robust multiarray average method, which takes into consideration the GC content. They were then analyzed using the Benjamin-Hochberg false discovery rate and filtered for confidence using a P value of <0.05 and for a change in expression greater or less than 1.5-fold. By employing the kits and analytical method described above for RT-PCR, several genes detected in this analysis were used to perform a semiquantitative RT-PCR analysis in order to confirm the GeneSpring results (see Fig. S1 and Table S1 in the supplemental material).

Statistics. Statistical significance was evaluated using the Student t test (two tailed, assuming unequal variance) in the Excel software program.

RESULTS

Exposure of ES cells to bacterial enteric pathogens. Cultured murine ES cells were exposed independently to a number of pathogenic bacteria, including *S. enterica* serovar Typhimurium, *S. flexneri* 5a, and EPEC. Murine ES cells derived from 129/Sv/Ev mouse embryos were employed in these studies. These cells were routinely cultured in LIF- and FBS-containing medium without feeder cells. Under the feeder-cell-free culture conditions the ES cells grew well and formed distinctive cellular clusters. Expression of the Oct3/4 antigen, an embryonic transcriptional factor expressed in undifferentiated ES cells, in propagated ES cells was monitored using flow cytometry. Initially, ES cells were compared to J774 murine macrophage-like cells. As shown in Fig. 1A, while more than 90% of the ES cells stained positively for Oct3/4 (Fig. 1A, panel A1), no cells were found to be positive when they were stained with antibodies for CD11b (Fig. 1A, panel A2). In contrast, as expected, 40% of J774 cells were weakly positive for Oct3/4 (Fig. 1A, panel A3), but 90% of the cells were positive for CD11b (Fig. 1A, panel A4).

To assess the level of bacterial invasion of ES cells, *S. enterica* serovar Typhimurium SL1344 and *S. flexneri* M90T Sh53 were transformed with plasmid pJKD18, which constitutively directs expression of the GFP, and transconjugant derivatives were utilized to infect ES cells. The presence of fluorescent intracellular bacteria was monitored by flow cytometry, using the FITC channel. As uninfected cells emitted a very low level of a detectable green fluorescent signal (Fig. 1B, panel B1, upper right quadrant), we concluded that any increased green fluorescent signals were due exclusively to expression of GFP from intracellular bacteria. The Oct3/4 expression of the same infected cell population was also monitored, and approximately 95% of the cells were found to be positive (Fig. 1B, panel B1, lower right quadrant). Two hours after infection with *S. flexneri* M90T Sh53(pJKD18), about 20% of the ES cell population was infected (Fig. 1B, panel B2, upper left quadrant). The values for *S. enterica* serovar Typhimurium SL1344(pJKD18)-infected ES cells were similar (data not shown). Further intracellular staining of the infected ES cell populations for Oct3/4 (PE or red channel) indicated that many of the cells that contained green bacteria also were positive for this marker (Fig. 1B, panels B3 and B4, upper right quadrants), indicating that bacteria infected undifferentiated cells. The level of Oct3/4 staining did not change with time postinfection (data not shown).

ES cells were also exposed to GFP-positive EPEC strain 31-6-1(pJKD18), and after fixation and permeabilization the cells were stained with DAPI (blue) (Fig. 1C, panel a) and actin (red) (Fig. 1C, panel c). EPEC strain 31-6-1(pJKD18) was formed clear fluorescent actin stain-positive lesions (Fig. 1C, panels a to d) that were indistinguishable from the lesions detected in epithelial cell lines, such as Hep2 cells (Fig. 1C, panel e). Thus, ES cells are apparently sensitive to invasion by *Salmonella* and *Shigella* and to formation of attaching and effacing lesions by EPEC.

TTSS-dependent ES cell invasion. In order to assess the contributions of TTSSs to ES cell invasion, we employed mutant derivative bacteria which were defective in TTSS structural components or effector proteins. The *S. flexneri* M90T *mxiD* mutant is defective in the *Shigella* TTTS translocon (1). The *S. enterica* serovar Typhimurium SL1344 *sipB* mutant is defective in formation of the SPI1 translocon, and the SL1344 *sopE* mutant is defective in expression of the SopE effector protein (21, 39). ES cells were infected with either *S. enterica* serovar Typhimurium SL1344 or *S. flexneri* M90T or mutant derivatives of these strains in standard gentamicin killing assays in which only internalized bacteria survived. When *S. enterica* serovar Typhimurium SL1344 was used, there was a steady increase in the number of viable internalized bacteria over the first 4 h and then a rapid increase in the number of intracellular bacterial between 4 and 5 h postinfection. At all time points after 3 h there was a statistically significant difference ($P < 0.005$ or $P < 0.05$) in the number of viable bacterial recovered compared with the number recovered at the 1-h time point (Fig. 2a). At the 2-h time point, mutations in *mxiD* and *sipB* almost completely eliminated *S. flexneri* M90T (Fig. 2b) ($P < 0.05$) invasion of ES cells and *S. enterica* serovar Typhimurium SL1344 (Fig. 2c) ($P < 0.05$) invasion of ES cells, respectively. A mutation in *sopE* reduced *S. enterica* serovar Typhimurium SL1344 invasion to a level that was 40% of the wild-type level (Fig. 2c) ($P < 0.05$), which is consistent with previous reports indicating that the TTSS-mediated invasion process is not fully dependent on *sopE* (39, 40).

Salmonella and *Shigella* can induce cell membrane extensions called ruffles upon entry into nonphagocytic cells through the action of their TTSSs (8). We examined bacterium-ES cell interactions by using SEM. Approximately 30 min after exposure of ES cells to *S. flexneri* M90T, thin, long filopodia which established initial contacts with the bacteria were readily visible on the ES cell surface associated with the bacteria (Fig. 3A, panel A1), and occasionally ES cells bearing bacteria enveloped in membrane folds were observed (Fig. 3A, panel A2). After the same time interval, cell membrane ruffles were frequently observed on *S. enterica* serovar Typhimurium SL1344-infected ES cells, and these ruffles were associated predominantly with one pole of the bacteria (Fig. 3B, panels B1 and B2). These membrane-associated structures differed somewhat from the structures in previously published SEM images obtained following *S. enterica* serovar Typhimurium interaction with cultured MDCK cells, in which the membrane extensions were much longer and whole bacteria were engulfed by the ruffles (18). Furthermore, *S. enterica* serovar Typhimurium SL1344 also appeared to be able to exploit filopodia and lamellipodia as sites of entry (Fig. 3B, panel B3). In contrast to the observations described above and although many cells were

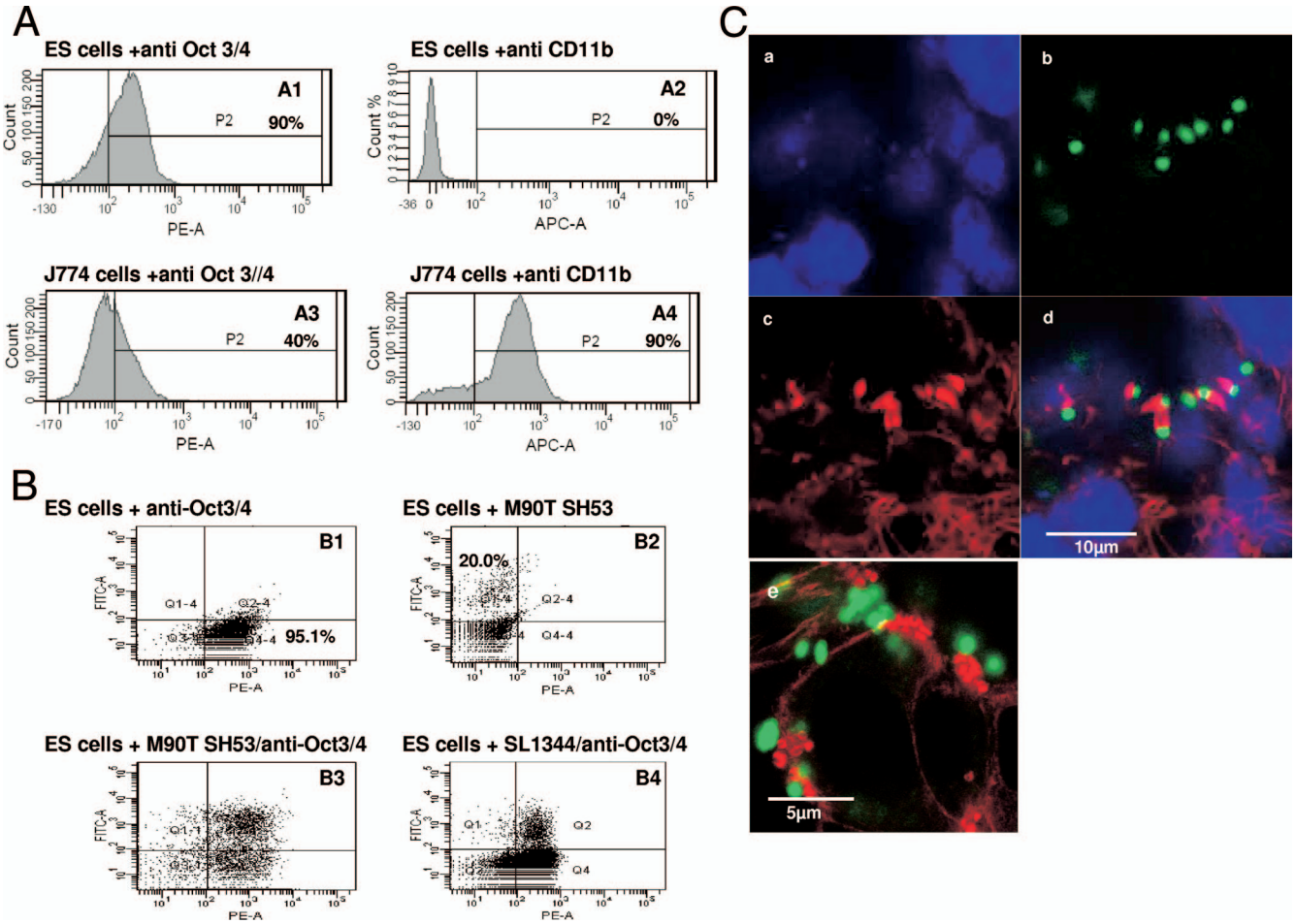

FIG. 1. (A) Detection of Oct3/4 by flow cytometry. (Panel A1) Approximately 90% of 2-day-old ES cells from feeder-cell-free culture were positively stained (P2, 90%) by anti-Oct3/4 conjugated with PE. (Panel A2) No ES cells from the same culture were stained by anti-mouse CD11b conjugated with APC. (Panels A3 and A4) Forty percent and 90% of the mouse J774 macrophage-like cells were found to be positive for Oct3/4 and CD11b, respectively. (B) Detection of Oct3/4 after bacterial invasion. ES cells were exposed to *S. flexneri* M90T(pJKD18) or *S. enterica* serovar Typhimurium SL1344(pJKD18) (MOI, 100), and cells were processed for flow cytometric analysis 2 h after infection. (Panel B1) Over 90% of uninfected cells were positively stained by anti-Oct3/4 antibody conjugated with PE (lower right quadrant), while no cells produced signals in the FITC channels (upper left quadrant). (Panel B2) Cells were infected by *S. flexneri* M90T Sh53(pJKD18) (green), and about 20% of the cells produced signals for bacteria in the FITC channel (upper left quadrant). (Panels B3 and B4) Large proportions of *S. flexneri* M90T Sh53(pJKD18)- and *S. enterica* serovar Typhimurium SL1344(pJKD18)-infected (green) cells were stained positively by anti-Oct3/4 antibody (upper right quadrant). (C) Confocal microscopy of EPEC 31-6-1(pJKD18)-infected (green) ES cells (panels a to d) or Hep2 cells (panel e). The ES cell nuclei and actin were stained with DAPI (blue) and Texas Red phalloidin (red), respectively. DAPI was not included in the Hep2 cell samples. Actin accumulation is apparent at the sites where EPEC 31-6-1 cells are attached. (Panel a) DAPI-stained ES cell nuclei. (Panel b) GFP-expressing EPEC 31-6-1. (Panel c) Texas Red-stained ES cell actin. (Panel d) Merged image for panels a, b, and c. (Panel e) Merged image for Hep2 actin and EPEC 31-6-1 staining.

examined by SEM, after exposure to the *S. enterica* serovar Typhimurium SL1344 *sopE* and *S. flexneri* M90T *mxiD* mutants (data not shown) the ES cells were found to have fewer membrane disruptions than ES cells exposed to wild-type *S. enterica* serovar Typhimurium SL1344 and *S. flexneri* M90T. Additionally, *S. enterica* serovar Typhimurium SL1344 bacteria were also frequently visible in indentations in the ES cells (Fig. 3B, panel B4), while *sopE* mutant bacteria were occasionally observed (Fig. 3B, panel B5) and *sipB* mutant bacteria were rarely observed (Fig. 3B, panel B6) in the same situation.

Expression of SPI2-associated promoters and subcellular localization. To monitor the expression of SPI2-associated genes, we infected ES cells with *S. enterica* serovar Typhimurium SL1344 transformed with plasmid pSsaG, which directs expression of GFP under control of the SPI2-associated promoter *ssaG* (31). We did not detect a distinct GFP signal when *S. enterica* serovar Typhimurium SL1344(pSsaG) was cultured in vitro in medium or 40 min after infection of ES cells (data not shown). However, by 2 h postinfection and in the presence of gentamicin, approximately 33% of ES cells were infected, as judged by flow cytometry (Fig. 4A, panel a). Also, the mean fluorescence intensity had increased from the value for uninfected cells (20 U) to ~468 U. At 4 h postinfection, the percentage of cells infected had increased only slightly to 37% (Fig. 4A, panel b), whereas the mean fluorescence intensity had increased to 1,293 U. These values demonstrate that the

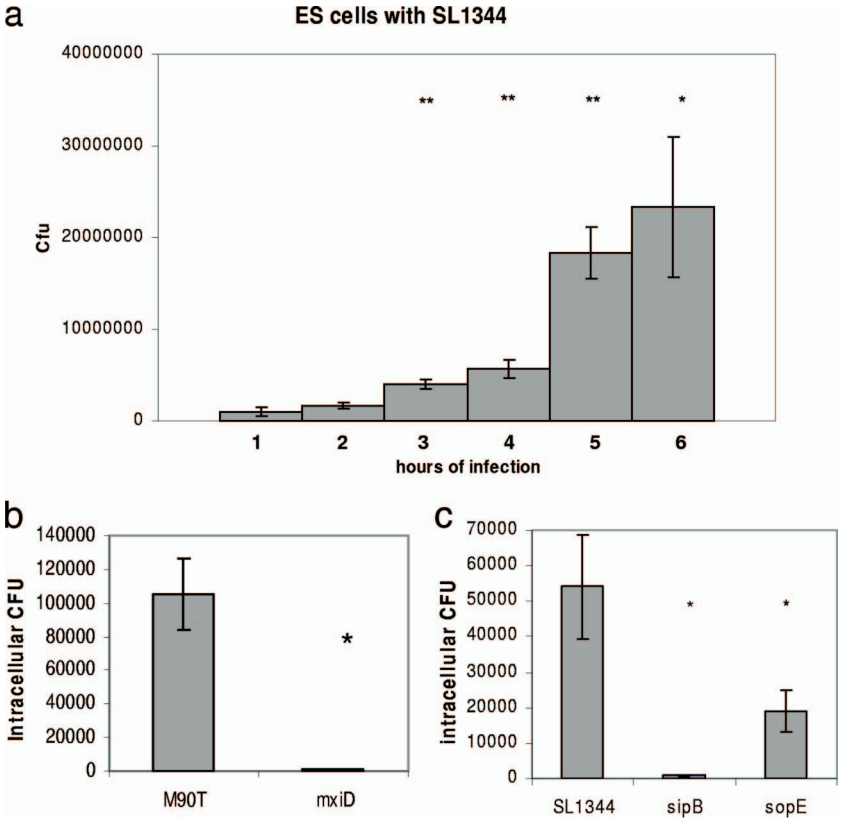

FIG. 2. Gentamicin killing assay. The bars indicate the numbers of intracellular CFU recovered over 1 to 6 h from ES cells infected with *S. enterica* serovar Typhimurium SL1344 (a) or 2 h after infection with *S. flexneri* M90T or with a *mxiD* mutant derivative (b) or with *S. enterica* serovar Typhimurium SL1344 or its *sopE* and *sipB* mutants (c) (MOI, 10). The error bars indicate standard deviations. ES cells were infected with bacteria for 30 min, washed with PBS, and then incubated in medium containing gentamicin (50 μg/ml) for 1 to 6 h prior to lysis. Cell lysates were plated in triplicate and incubated overnight at 37°C, and the resulting colonies were counted. Student *t* tests were performed to compare the numbers of CFU recovered at all time points with the number of CFU recovered at the 1-h time point (*, $P < 0.05$; **, $P < 0.005$).

ssaG promoter is specifically activated inside ES cells, possibly in the *Salmonella*-containing vacuole (SCV), and that while in the presence of gentamicin the percentage of cells infected does not change significantly, the level of GFP expression does.

To obtain evidence showing that *Salmonella* was in an SCV in ES cells, we stained *S. enterica* serovar Typhimurium SL1344-infected ES cells with antibodies against *Salmonella* common surface antigen (CSA) conjugated to tetramethyl rhodamine isocyanate and lysosome-associated membrane protein 1 (Lamp-1) linked to FITC and examined the preparations using confocal microscopy. As a predicted negative control, *S. flexneri* M90T-infected ES cells were stained with anti-*Shigella* lipopolysaccharide-Texas Red and anti-Lamp-1–FITC since *S. flexneri* normally occurs free in the cell cytosol after escape from the vacuole (8). *S. enterica* serovar Typhimurium SL1344 bacteria were indeed observed to be predominantly colocalized with Lamp-1 within ES cells 2 h after infection (Fig. 4B), while the intracellular *S. flexneri* M90T bacteria were not, as expected (Fig. 4C). Surprisingly, despite the fact that all slides were stained simultaneously, the level of Lamp-1 staining was always noticeably lower in M90T-infected cells than in *Salmonella*-infected cells, as shown in Fig. 4B and 4C. Attempts to colocalize *Salmonella* at the 2-h time point with early endosomal markers, such as EEA-1, the transferrin receptor CD71, or Rab7, all failed. However, based on the findings of Knodler and Steele-Mortimer (23), this is not surprising as the colocalization events are extremely early events and probably occur before the first time point that we used (1 h). Further evidence obtained by using TEM showed that *S. enterica* serovar Typhimurium SL1344 bacteria indeed occurred predominantly within membrane-bound vacuoles (Fig. 4D, panel D1), which were occasionally observed to be fused with lysosomes (Fig. 4D, panel D2). In contrast, the *S. flexneri* M90T bacteria observed were frequently not associated with obvious vacuole-like structures as determined by TEM (Fig. 4D, panel D3). Further, using fluorescent reagents, some intracellular *S. flexneri* M90T bacteria were found to form actin tails at one pole (Fig. 4D, panel D4), which is known to be mediated by the surface protein IcsA (4). These results strongly support the idea that *S. enterica* serovar Typhimurium resides within an SCV in ES cells and demonstrate that *S. flexneri* can be found in the ES cell cytosol, as observed for other cell types (8).

Cell death after *Salmonella* and *Shigella* infection. *Salmonella* and *Shigella* are known to have the potential to infect many different types of cells, although the host responses, such as apoptosis, can differ depending on the type of host cells infected (13, 17, 19, 30, 41). To assess ES cell death during *S. enterica* serovar Typhimurium and *S. flexneri* infection, we

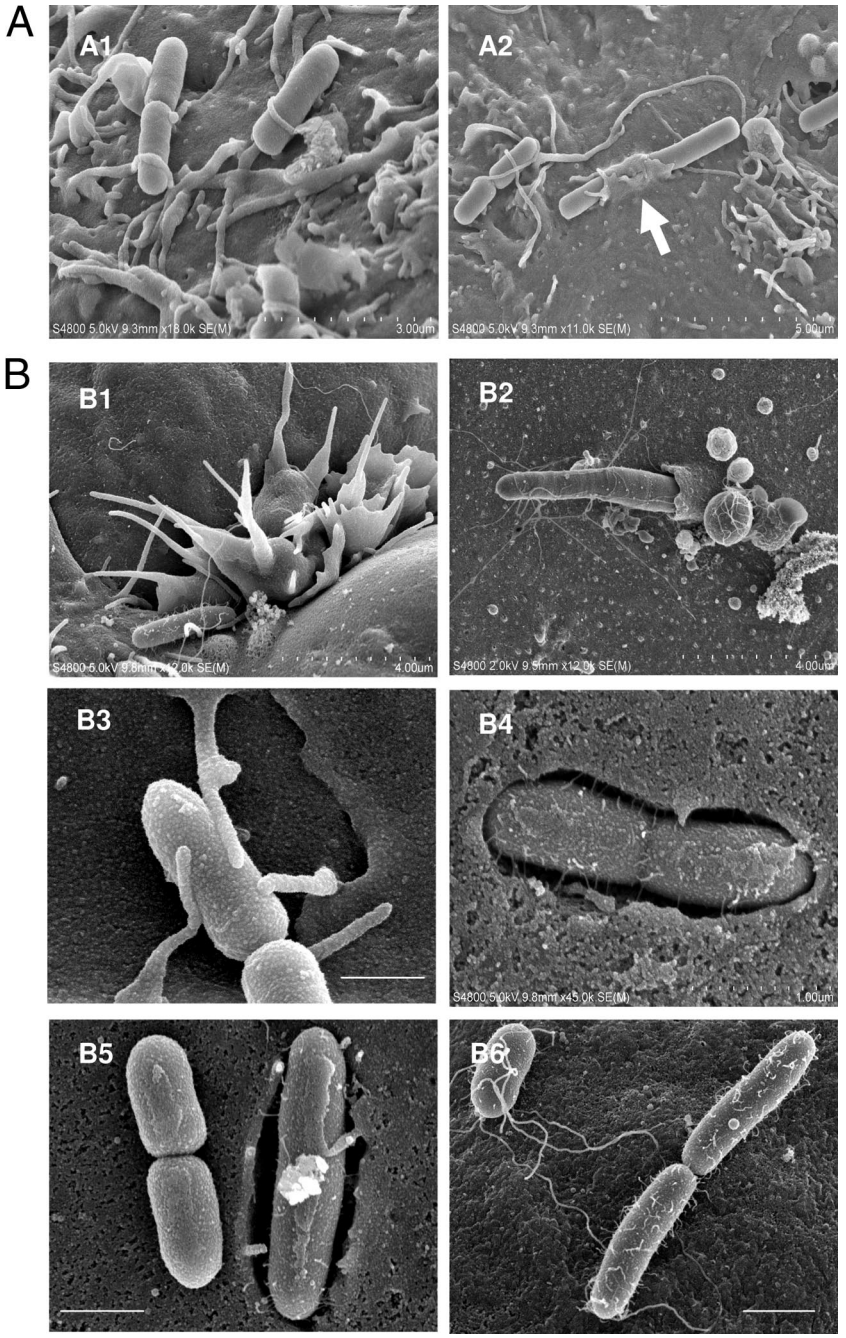

FIG. 3. SEM of ES cells infected by *S. flexneri* M90T (A) or by *S. enterica* serovar Typhimurium SL1344 or *S. enterica* serovar Typhimurium SL1344 *sopE* and *sipB* mutants for 30 min (B). (A) *S. flexneri* cells are apparently entangled in thin, long ES cell filopodia (panel A1), and one dividing *S. flexneri* cell is wrapped up by cell membrane (panel A2, arrow). (B) (Panels B1 and B2) Two examples of *S. enterica* serovar Typhimurium-induced ES cell membrane extensions or ruffles. (Panel B3) *S. enterica* serovar Typhimurium can exploit filopodia or lamllipodia as entry ports. (Panel B4) *S. enterica* serovar Typhimurium SL1344 bacterium that apparently sank into a cell without induction of apparent ruffles. (Panels B5 and B6) Uptake of *S. enterica* serovar Typhimurium SL1344 *sopE* and *sipB* mutants, respectively.

monitored cell viability using a CellTiter assay in which the quantitative luminescent signals generated by luciferase activity reflect the levels of ATP released from viable cells. With this assay we did not detect significant differences between infected and uninfected ES cells up to 4 h postinfection (data not shown), although by 24 h there was evidence of killing of the whole cell culture as determined by light microscope observation.

Subsequently, we screened for apoptotic ES cells by using confocal microscopy combined with a Sulforhodamine FLICA kit. This kit contained the reagent SR-VAD-FMK, which covalently binds to activated caspases. The murine macrophage-

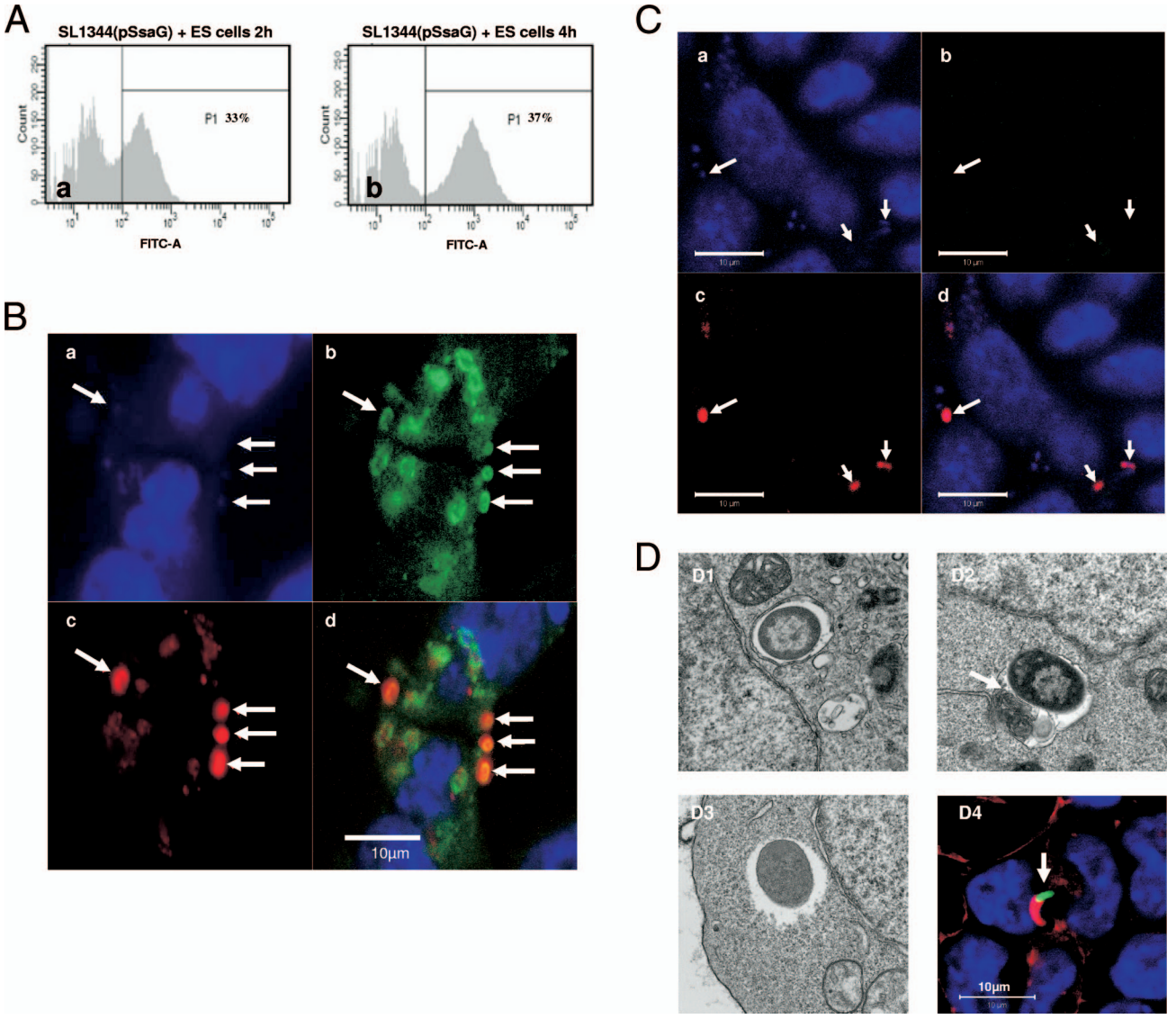

FIG. 4. (A) Flow cytometric analysis of *S. enterica* serovar Typhimurium SL1344(pSsaG)-infected ES cells (MOI, 100) 2 h (panel a) and 4 h (panel b) postinfection. The percentages of the infected cell population (P1) are indicated, and the mean fluorescence intensities at 2 and 4 h postinfection were 468 and 1,293 U, respectively. (B and C) Confocal microscopy of intracellular *S. enterica* serovar Typhimurium SL1344 and *S. flexneri* M90T, respectively. (Panels a) Cell nuclei stained with DAPI (blue). (Panels b) Lamp-1 stained by an anti-Lamp-1 monoclonal antibody conjugated with FITC (abCam). (Panels c) *S. enterica* serovar Typhimurium stained red utilizing goat anti-*Salmonella* CSA (Kirkegaard & Perry Laboratories) and donkey anti-goat immunoglobulin G conjugated with tetramethyl rhodamine isocyanate (Jackson) and *S. flexneri* M90T stained with rabbit anti-*Shigella* lipopolysaccharide and goat anti-rabbit immunoglobulin G conjugated with Texas Red (abCam). (Panels d) Merged images for panels a, b, and c. The arrows in panel B indicate colocalized *S. enterica* serovar Typhimurium SL1344 and Lamp-1, and the arrows in panel C indicate intracellular *S. flexneri* M90T that was not associated with Lamp-1. (D) TEM (panels D1, D2, and D3) and confocal (panel D4) images of *S. enterica* serovar Typhimurium SL1344- and *S. flexneri* M90T-infected ES cells. (Panel D1) Intracellular *S. enterica* serovar Typhimurium SL1344 bacterium in a membrane-bound vacuole. (Panel D2) *S. enterica* serovar Typhimurium SL1344 bacterium containing a vacuole fused with a lysosome (arrow). (Panel D3) Intracellular *S. flexneri* M90T bacterium free in the cell cytosol. (Panel D4) Confocal image of an intracellular *S. flexneri* M90T(pJKD18) bacterium with an actin tail behind one pole (arrow). To observe *S. flexneri* M90T(pJKD18)-directed actin tails, ES cells were fixed and stained with Texas Red phalloidin as described in Materials and Methods.

like J774 cell line, which is known to undergo apoptosis upon *Salmonella* infection, was used as a control. Two hours postinfection most of the J774 cells observed were infected by *S. enterica* serovar Typhimurium SL1344(pJKD18) (green) and were stained with SR-VAD-FMK (red) (Fig. 5A). In contrast, activated caspases were not detected by the same experimental procedure in any ES cells, including those infected by *S. enterica* serovar Typhimurium SL1344(pJKD18) (Fig. 5B). Further attempts to demonstrate apoptotic events in naïve and infected ES cells using other methods, including ApoDIRECT in situ DNA fragmentation analysis, annexin V staining, Biovision CaspGLOW red anti-caspase detection, and CaspSCreen staining, were all unsuccessful.

In a final attempt to establish the levels of caspase expres-

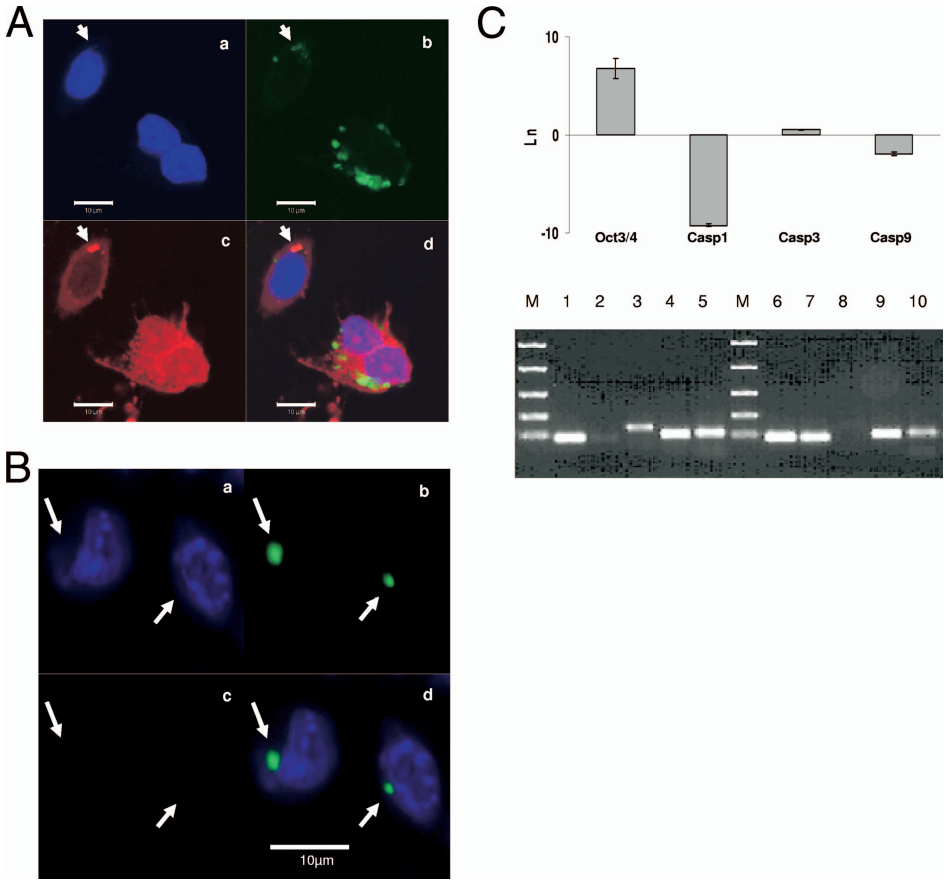

FIG. 5. (A and B) Detection of activated caspases by confocal microscopy combined with SR-VAD-FMK staining. J774 and ES cells were infected for 2 h (MOI, 100) with *S. enterica* serovar Typhimurium SL1344(pSsaG), which expressed GFP only when it was inside host cells. (Panels a) Cell nuclei were stained by DAPI (blue). (Panels b) Intracellular GFP-expressing bacteria. (Panels c) SR-VAD-FMK-stained intracellular activated caspases. (Panels d) Merged images for panels a, b, and c. Bars = 10 μm. In panel A, two connected J774 cells have extensive accumulation of granular forms of caspases and also appear to shed caspases to the extracellular milieu, and a single intact cell has only one large mass of activated caspases, which is colocalized with an *S. enterica* serovar Typhimurium SL1344(pSsaG) bacterium weakly expressing GFP (arrow). In panel B, all ES cells, both infected (arrows) and uninfected, were hardly stained by SR-VAD-FMK. (C) Detection of Oct3/4 transcripts by real-time RT-PCR. The differences in levels of expression between ES and J774 cells are indicated by $2^{\wedge}(-\Delta\Delta C_T)$ values on natural log scale; values greater than and less than zero indicate higher and lower levels of expression in ES cells than in J774 cells, respectively. To visualize the RT-PCR products, 10 μl of each reaction mixture was loaded on a 1% agarose gel, and all PCR products migrated between the 100- and 250-bp markers. Lanes M, EasyLadder DNA size markers (Bioline); lanes 1 and 6, β-actin from J774 and ES cells, respectively; lanes 2 and 7, Oct3/4 from J774 and ES cells, respectively; lanes 3 and 8, caspase-1 from J774 and ES cells, respectively; lanes 4 and 9, caspase-3 from J774 and ES cells, respectively; lanes 5 and 10, caspase-9 from J774 and ES cells, respectively.

sion, we performed real-time RT-PCR using total RNA from ES cells and control J774 cells. The results showed that compared to J774 cells, ES cells contained higher levels of the Oct3/4 transcript but no detectable caspase-1 transcript (Fig. 5C, upper panel). The differences in the contents of the caspase-3 and caspase-9 transcripts between ES and J774 cells were not significant. The data for the real-time RT-PCR products (Fig. 5C, lower panel) clearly showed that ES cells produced low levels of caspase-1 mRNA than J774 cells produced (Fig. 5C, lower panel, compare lane 8 with lane 3), but they did produce comparable levels of caspase-3 and caspase-9 mRNA (Fig. 5C, lower panel, compare lane 9 with lane 4 and lane 10 with lane 5). Additionally, low levels of Oct3/4 transcripts were detected in J774 cells (lane 2), which agreed with the flow cytometry data described above (Fig. 1A, panel A3).

Affymetrix microarray expression profile analysis of ES cells during infection. In order to obtain further insight into ES cell-bacterium interactions, Affymetrix murine microarrays (MIAMExpress accession number E-MEXP-1862) were employed to analyze gene expression patterns in ES cells 2 and 4 h after infection with *S. enterica* serovar Typhimurium SL1344(pSsaG). The expression data were analyzed by comparing pairs using the GeneSpring platform (www.chem.agilent.com). Compared to the uninfected cells, there were only 19 genes that were significantly ($P < 0.05$ and 1.5-fold change) up- or downregulated at 2 h postinfection and 89 genes (56 of which were previously annotated) that were significantly up- or downregulated at 4 h postinfection (see Tables S1 and S2 in the supplemental material). The mRNA expression profile at 4 h postinfection was also compared to the expression profile at 2 h postinfection. This comparison revealed 87 genes that were significantly up- or downregulated, 63 of which were previously annotated (see Table S3 in the supplemental material). Interestingly, cytochrome P450 was

first upregulated at 2 h infection (see Table S1 in the supplemental material) and was strongly downregulated at 4 h infection (see Table S2 in the supplemental material). Among the genes differentially regulated were genes associated with stress responses or cell cycle-regulating genes, including Ccng2 and Banp, while an antiapoptotic gene, Hspa1b (Hsp70), was strongly upregulated. The latter gene is also involved in stress responses and in negative regulation of caspase activity (positive regulation of antiapoptosis) plus the NF-κB-mediated inflammatory response (35). A few genes coding for transcription factors were revealed, and one of these genes, X Box Protein member 1 (Xbp1), was consistently upregulated. Xbp1 has been described as a factor that is involved in antigen-presenting cell differentiation and the endoplasmic reticulum stress response (22, 32). Genes involved in the cellular response to bacterial invasion were detected; however, no genes obviously involved in innate immunity or apoptosis were differentially expressed. ES cells are known to tightly regulate gene expression in order to maintain pluripotency and avoid entering a differentiated state without receiving the appropriate signal, and factors such as the proteosome have been implicated in maintaining this tight transcriptional regulation (37). Our array data suggest that infection by bacterial pathogens is not sufficient to significantly disrupt this state under the conditions that we employed.

Role for cholesterol synthesis in *Salmonella* and *Shigella* infection of ES cells. To demonstrate the feasibility of using mutant ES cell lines, we performed experiments to determine if cellular cholesterol levels are important for *S. enterica* serovar Typhimurium and *S. flexneri* invasion of ES cells. Eukaryotic cell plasma membranes can harbor cholesterol- and sphingolipid-rich domains, termed lipid rafts, which have been proposed to be targeted by both *Salmonella* and *Shigella* for delivering effector proteins into host cells (5, 21, 26). Initially, ES cells were treated with methyl-β-cyclodextrin (MbCD), which is a water-soluble saccharide that is able to form inclusion complexes with cholesterol, hence limiting any potential interaction of cholesterol with the bacterial translocon (21). For bacterial invasion by either *S. enterica* serovar Typhimurium SL1344 (Fig. 6A, panel a) or *S. flexneri* M90T (Fig. 6A, panel b) there was a clear dose-dependent decrease with MbCD concentration, and infection was nearly abolished when a concentration of MbCD greater than 2 mM was used (for SL1344, 2 and 3 mM [$P < 0.0005$]; for M90T, 3 mM [$P < 0.05$]).

We then used a genetic approach to investigate whether a reduction in ES cell cholesterol levels could have an impact on the efficiency of bacterial invasion. We acquired a mutant 129S2 (formerly 129/SvPas) ES cell line, M064F03, which has a gene trap in the promoter region of the Sqle gene that encodes squalene monooxygenase, a rate-limiting enzyme required for catalysis of the first oxygenation step in sterol biosynthesis. Using the fluorescent reagent filipin, which binds cholesterol, we observed consistently lower cell surface cholesterol levels in M064F03 cells than in the TBV2 parent cell line (data not shown). In agreement with this, using RT-PCR, we also found that M064F03 cells produced significantly lower levels of Sqle transcripts than TBV2 cells produced (Fig. 6B). Thus, although the gene trap inactivated only one allele, this mutation significantly affected Sqle gene expression through reduced transcription.

We then performed comparative gentamicin killing assays (Fig. 6C) with mutant M064F03 and control TBV2 ES cells using the *S. enterica* serovar Typhimurium SL1344 (Fig. 6C, panel a) and SL1344 *sopE* (Fig. 6C, panel b) and SL1344 *sipB* (Fig. 6C, panel c) strains. After enumeration of the viable bacteria recovered at time points over a 5-h period, it was clear that M064F03 cells were less able to support an *S. enterica* serovar Typhimurium SL1344 infection ($P < 0.05$, $P < 0.005$, and $P < 0.0005$) than wild-type TBV2 cells. Gentamicin assays performed utilizing *S. flexneri* also demonstrated that the M064F03 mutant cell line was statistically significantly less susceptible to invasion (Fig. 6D) than the TBV2 parent line ($P < 0.0005$ at 2 and 4 h; $P < 0.005$ at 3 h). Electron micrographs (2 h) (Fig. 6E, panels A to F) of *S. enterica* serovar Typhimurium SL1344-infected ES cells indicated that, while the bacteria did invade both types of cells, the cholesterol mutant M064F03 ES cells (Fig. 6E, panels A to C) developed vacuoles with multiple membranes (Fig. 6E, panel A), while double-membrane-bound SCV were rarely detected in TBV2 cells (Fig. 6E, panel E). Furthermore, tubulin-like structures were observed not only in the vicinity of SCV (Fig. 6E, panel C) but also enclosed in the SCV in M064F03 cells (Fig. 6E, panel A). The vacuoles in both types of cells frequently fused with lysosomes (Fig. 6E, panels B and F) as described above (Fig. 4D, panel D2). Confocal images (4 h) of infected cells stained for Lamp-1 and *Salmonella* CSA (Fig. 6E, panels G to L) also demonstrated that the SCV in both types of cells colocalized with late endosomal marker Lamp-1, as shown in Fig. 4B, but we suggest that the distribution in the M064F03 cells is less defined (Fig. 6E, panel H) than that in TBV2 cells (Fig. 6E, panel K). If the M064F03 SCV were aberrant, it could allow access of the lysosome contents to the bacteria, thus explaining the different numbers of viable bacteria recovered (Fig. 6C). However, further studies are required to investigate this hypothesis. Filipin staining (red) (Fig. 6F, panels A and D) of both types of cells, in combination with anti-CSA FITC staining (green) (Fig. 6F, panels B and E) of the bacteria, confirmed that cholesterol was sequestered around the bacteria in both types of cells (Fig. 6F, panels C and F).

DISCUSSION

Murine undifferentiated ES cells, which have a normal karyotype, are not transformed, and maintain pluripotency in culture, provide a cell-based system that is an alternative to terminally differentiated cells for infection studies. However, little is known about how cultured ES cells interact with bacteria. Here we report the results of the first investigations of murine ES cells and show that these cells can be infected independently with *S. flexneri*, *S. enterica* serovar Typhimurium, and EPEC. Flow cytometric analysis demonstrated that a majority of infected ES cells were maintained in the primitive undifferentiated state when they were cultured under feeder-cell-free culture conditions. The expression of low levels of Oct3/4 in the control J774 cells was not unexpected as some transformed cells are known to possess stem cell characteristics (10). Oct3/4 transcripts were also detected in J774 cells by RT-PCR. In contrast, no undifferentiated ES cells were

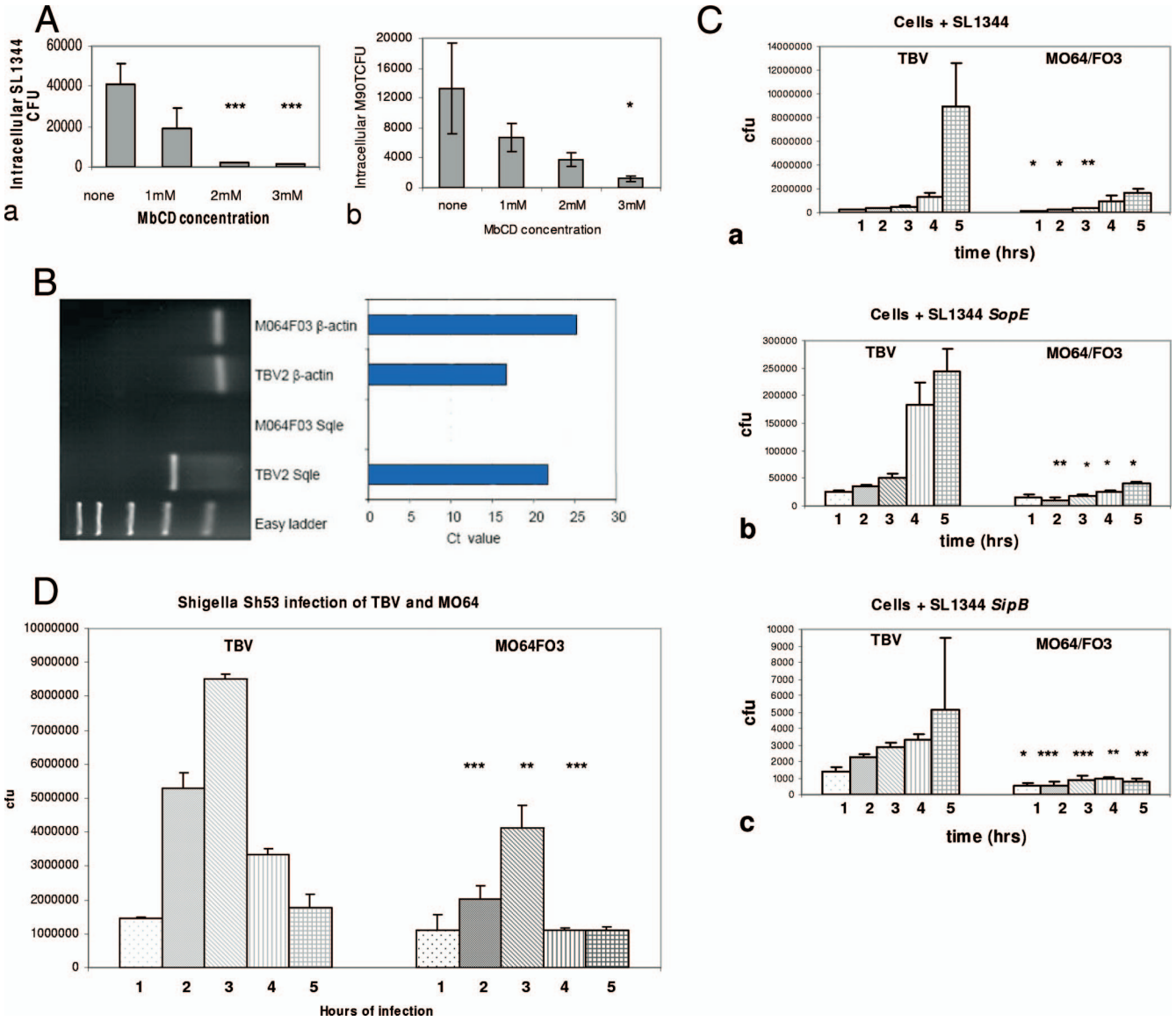

FIG. 6. Influence of cell cholesterol level on *S. enterica* serovar Typhimurium and *S. flexneri* infection. (A) Gentamicin killing assay with ES cells that were treated with MbCD and infected with *S. enterica* serovar Typhimurium SL1344 (panel a) and *S. flexneri* M90T (panel b). Cells were treated with MbCD at the indicated concentrations for 40 min and washed twice with PBS before addition of bacterial suspensions to the cells (MOI, 10), which was followed by 2 h of incubation under gentamicin selection conditions. Student *t* tests were performed to compare the numbers of CFU recovered with all MbCD concentrations with the numbers of CFU of the control cells (*, $P < 0.05$; ***, $P < 0.0005$). (B) Detection of Sqle gene transcripts by real-time RT-PCR. The difference in the levels of expression are indicated by C_T values for Sqle and β-actin genes from each cell line. Note that the C_T value for the Sqle transcript from M064F03 cells was zero, indicating that there was no Sqle gene expression. (C) Gentamicin killing assays with *S. enterica* serovar Typhimurium SL1344-infected (panel a), SL1344 *sopE* mutant-infected (panel b), or SL1344 *sipB* mutant-infected (panel c) TBV2 and M064/F03 ES cells. Infection was carried out for 1 to 5 h (MOI, 10). ES cells were infected with bacteria for 30 min, washed with PBS, and then incubated in medium containing gentamicin (50 μg/ml) for 1 to 5 h prior to lysis. Cell lysates were plated in triplicate and incubated overnight at 37°C, and the resulting colonies were counted. Student *t* tests were performed to compare the number of CFU recovered from M064/F03 cells at each time point with the number of CFU recovered at the same time point from TBV2 cells (*, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.005$). (D) Gentamicin killing assay with *S. flexneri* Sh53 and TBV2 and M064/F03 ES cells. Cells were infected with *S. flexneri* (MOI, 10) for 30 min, washed with PBS, and then incubated in medium containing gentamicin (50 μg/ml) for 1 to 5 h prior to lysis. Cell lysates were plated in triplicate and incubated overnight at 37°C, and the resulting colonies were counted. Student *t* tests were performed to compare the number of CFU recovered from M064/F03 cells at each time point with the number of CFU recovered from TBV2 cells at the same time point (**, $P < 0.005$; ***, $P < 0.0005$). (E) TEM images (panels A to F) and confocal images (panels G to L) of *S. enterica* serovar Typhimurium SL1344-infected M064/F03 and TBV2 cells. (Panels A to C) SCV in M064/F03 cells 2 h postinfection. The thin arrows indicate tubulin-like structures (panels A and C), and the wide arrows indicate a fused lysosome (panel B) and a single membrane (panel C). (Panels D to F) SCV in TBV2 cells 2 h postinfection. The arrowheads indicate a double membrane (panel E), and the wide arrows indicate lysosomes (panel F). The confocal images are images of M064/F03 cells (Panels G to I) and TBV2 cells (panels J to L) at 4 h postinfection stained with anti-*Salmonella* CSA directly conjugated to FITC (panels G and J) and stained with rat anti-Lamp-1 and counterstained with anti-rat immunoglobulin-APC Cy7 (panels H and K) and merged images (panels I and L). The thin arrows indicate suggested different patterns of staining. (F) Confocal images of M064/F03 (panels A to C) and TBV2 (panels D to F) cells 2 h after infection with *S. enterica* serovar Typhimurium SL1344 stained with filipin (panels A and D) and anti-*Salmonella* CSA directly conjugated to FITC (panels B and E) and merged images (panels C and F).

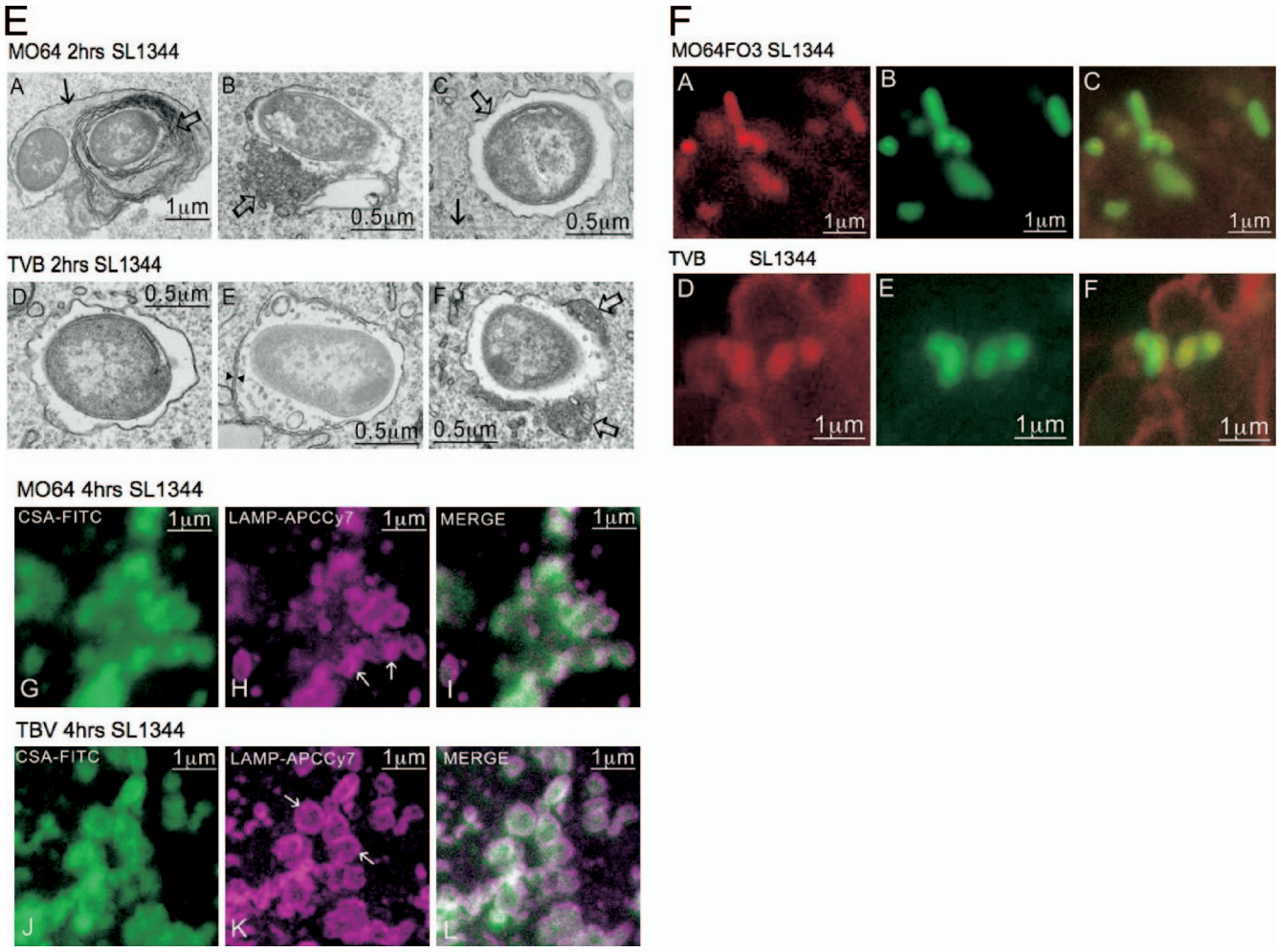

FIG. 6—*Continued.*

found to be positive for CD11b, a marker antigen normally expressed on macrophage cell surfaces. Also, by using flow cytometric analysis we were able to confirm infection of murine ES cells by GFP-expressing bacteria and then, using further internal staining of the infected ES cells for Oct3/4, show that the bacteria infected undifferentiated ES cells and that such cells remained in this state throughout the infection assay.

As GFP is a long-lived protein and therefore once it is expressed its presence does not indicate bacterial viability, we monitored the numbers of culturable intracellular *S. enterica* serovar Typhimurium SL1344 and *S. flexneri* M90T bacteria recovered from exposed ES cells using gentamicin assays in which extracellular bacteria were selectively killed. Both *S. enterica* serovar Typhimurium SL1344 and *S. flexneri* M90T efficiently invaded ES cells at levels slightly higher than those observed for control Hep2 cells, which are routinely used in such invasion assays. The number of recovered *S. enterica* serovar Typhimurium SL1344 bacteria increased over a 6-h period, as expected, with the largest increase in the number of bacteria occurring between 4 to 5 h, whereas *S. flexneri* M90T infection was more rapid and the number of bacteria declined by 4 h. Using gentamicin assay data and electron microscope observations at single time points for the TTSS *S. enterica* serovar Typhimurium *sopE* mutant (39, 40) and *sipB* or *S. flexneri mxiD* mutant (1) bacteria, it was possible to observe lower infection rates and reduced or no formation of ruffle structures. Considering these data together, we concluded that infection of ES cells by *S. enterica* serovar Typhimurium and *S. flexneri* is TTSS dependent, implying that there is an active invasion process rather than a passive macropinocytotic process, as observed previously for hematopoietic stem cells (24). Once inside conventional host cells, *S. enterica* serovar Typhimurium survives and proliferates in a specialized membrane compartment termed SCV through the action of a second TTSS encoded by SPI2 (25), while *Shigella* cells rapidly escape from the vacuole and reside in the cytoplasm. The results reported here indicate that the *ssaG* promoter utilized in our experiments with *S. enterica* serovar Typhimurium (*ssaG*) is specifically activated inside ES cells, resulting in increased GFP production, likely in the SCV. This finding is similar to data obtained previously using human macrophage-like U937 cells (31). We also confirmed that salmonellae, but not shigellae, once intracellular, are located at a position close to Lamp-1, as observed in conventional types of laboratory cells.

Utilizing a light microscope, cultured ES cells infected for 24 h showed clear detachment and death. However, use of

three different methods to reveal caspase activation (ApoDIRECT, CaspSCreen, and CaspGLOW from MBL) and use of an APC-conjugated antibody against the apoptotic marker annexin V did not reveal any positive apoptotic signal. Human ES cells are prone to spontaneous apoptosis and differentiation, which is likely due to activation of a mitochondrial pathway through p53 (34). This could occur during bacterial invasion of murine ES cells, an hypothesis potentially supported by our microarray analysis which showed that the banp (btg3-associated nuclear) gene was significantly upregulated. On the other hand, LIF has been shown to be an antiapoptotic mitogen for pluripotent mouse ES cells in a serum-free medium (16). The amount of LIF that we routinely added to the medium was probably sufficient to prevent ES cells from entering apoptosis even when the ES cells encountered bacteria such as *S. enterica* serovar Typhimurium and *S. flexneri*. Furthermore, the stem cell factor-KIT (a member of family III of receptor tyrosine kinases) signaling pathway and LIF were recently found to work synergistically to protect mouse ES cells from undergoing apoptosis. Withdrawing LIF can induce apoptosis only in stem cell factor-KIT null cells and not in wild-type ES cells (3). Additionally, *S. enterica* serovar Typhimurium and *S. flexneri* use *sipB* and *ipaB*, respectively, to induce apoptosis in macrophages and dendritic cells by activation of caspase-1 (19). However, only caspase-3 has been implicated to have a role in mouse ES cell apoptosis, either when cells have undergone neuronal differentiation (27) or upon treatment with ionizing radiation in the Unr (upstream of N-ras) null background (12). The RT-PCR analysis of caspase expression in murine macrophage cell line J774 and ES cells highlighted the lack of caspase expression in the latter cells. These results, together with microscope observations and the microarray analysis results, imply that if ES cells die during bacterial invasion, the process does not involve caspase activation. Microarray analysis revealed the absence of a significant immune response in infected the ES cell immunological reaction. These data agree with the results of our studies in which we looked for cytokine expression and the appearance of immune markers on ES cells (data not shown). Considering that the ES cells utilized in the study were undifferentiated, the lack of a strong immunological signal is not necessarily surprising.

Finally, in this study we investigated the applicability of mutant ES cells for analysis of host-pathogen interactions by using ES cells defective in cholesterol biosynthesis. Initially, to do this, parental ES cells were treated with MbCD to chelate cholesterol, and compared with untreated ES cells, these cells were found to be less supportive of *Salmonella* infection as the concentration of MbCD increased. Significantly, the Sqle mutant ES cell line M064F03, deficient in cholesterol synthesis, was less supportive of *S. flexneri* and *S. enterica* serovar Typhimurium infection than the parental ES cell line. Microscopic examination of infected Sqle mutant ES cells suggested that invading bacteria may become enmeshed in vacuoles harboring multiple membranes that may be more susceptible to fusion with lysosomal elements within the cell, leading to enhanced bacterial killing. The data obtained illustrate some of the potential for exploiting mutant ES cells in such studies of host-pathogen interactions.

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